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Summary

Roger Yonchien Tsien (1952-2016; Elected ForMemRS 2006) displayed precocious childhood talents in chemistry. After graduating from Harvard University in chemistry and physics in 1972, he pursued a PhD programme in the Physiological Laboratory, Cambridge, under Prof. Richard Adrian's supervision with Marshal Scholarship support. His thesis "The design and use of organic chemical tools in cellular physiology" won the Gedge Prize, and a Comyns Berkeley Research Fellowship by Gonville and Caius College supporting his postdoctoral work developing Ca^{2+}-sensitive electrodes and fluorescent probes measuring cellular [Ca^{2+}]. His novel tetracarboxylate quin-2 readily accessed cells as its subsequently cleavable acetomethoxy-ester and signalled in the physiological [Ca^{2+}] range. At Berkeley, further improved ratiometric and strongly fluorescent probes included the Ca^{2+}-sensing indo-1, fura-2 and fluo-3, sensors for other strategic ions, and molecules that could photolytically uncage key intracellular regulators. At San Diego, design of fluorescent protein complexes build from the cAMP-binding phosphokinase A was followed by Nobel prizewinning work expressing green fluorescent protein (GFP) and developing spectral GFP variants forming fluorescent resonance energy transfer (FRET) pairs. Their conjugation with further proteins specifically binding particular ions or molecules under study permitted measurement of these by optical methods. Further detector protein complexes such as DsRED, fluorescent at longer wavelengths were to complement GFP. These interests culminated in the development of chemical tools with potential diagnostic and therapeutic clinical applications through their optical and magnetic resonance signals. His scientific work bequeathed a lasting wealth of chemical tools invaluable for physiological investigation with both current and future applicability.

Early days and education

Roger Yonchien Tsien was born in New York City on February 1, 1952, the third of three sons to the MIT-trained mechanical engineer Hsue Chu Tsien, and the trained nurse, Yi Ying. His family lineage fell within the scholar-gentry Hangzhou tradition, with early historically recorded interactions with the Song Dynasty. His family background also had an unusual backdrop reflecting the Chinese historical events during and immediately following the Second World War. Their often exceptionally talented progeny often subsequently led impressive and imaginative careers in the West despite adversity and even prejudice. His father ran a small export-import business in New York City and an engineering consultancy firm in Westchester County. His father then moved his family to Livingstone, New Jersey, when Roger was aged 8, and there worked on vacuum tubes and petrochemicals with the Radio Corporation of America (RCA) and Esso Research and engineering respectively.

Roger displayed precocious childhood aptitudes for scientific exploration that developed into a passion for theoretical and practical benchside chemistry. This began presciently with interests in ligand-related chromophore behaviour of coloured transition metal ions, going on to a wide range of classic inorganic chemistry experiments, culminating in attempts to synthesize acetylsalicylic acid, often using improvised apparatus and glassware. These sometimes adventurous investigations were often performed, appropriately, in the family garden given their sometimes dramatic outcomes reminiscent of those in Humphrey Davy's experiments as a scientific prodigy. Unusually but unsurprisingly, Roger's first Boy Scout merit badge was in chemistry. His notebook of chemistry experiments as an 8-year-old is now preserved in the Nobel Museum in Stockholm, Sweden. His aptitude and fascination with chemistry continued into his school years. He pursued a National Science Foundation-sponsored summer research program at Ohio University in 1967 investigating amphidentate ligand properties of thiocyanate (SCN^-) arising from the similar distribution of negative charge between its nucleophilic sulphur and nitrogen. The latter offered the possibility of bond formation bridging two or more metals through the respective bonding of the component N and the S atoms with class A and B metals. What he perceived as a frustrating search nevertheless gained a first prize award in the Westinghouse Science Talent Search competition in 1968 when he was aged 16.

Roger then entered Harvard, intermitting his chemistry interests with its liberal programme offering opportunities in the humanities, and encounters with Walter Gilbert in molecular biology, and Jack Nichols, David Hubel and Torsten Weisel in visual and Nelson Kiang in auditory neurophysiology. He graduated *Phi Beta Kappa* in chemistry and physics in 1972, summa cum laude, winning the Detur Prize. Advice from these mentors prompted him to apply successfully for a Marshall Scholarship supporting a PhD programme in Churchill College in the University of Cambridge in the United Kingdom.

His Cambridge PhD supervisor, Professor Richard Adrian had research interests in the cell physiology of skeletal muscle activation differing from Rogers' own. However, both shared scientific and personal, values and modes of conduct, kind, generous and gracious attitudes and absolutely honest and humane treatment of others, and were both consummate scientists driving the limits of their scientific work. Richard Adrian was more interested in giving those of his students of high intellectual and scientific calibre a chance to develop their original drive and independence rather than amassing a collection of subordinates toiling uncritically under the direction of a principal investigator. Such was also the culture in the Physiological Laboratory (Hill, 1965), which doubtless contributed to its distinguished scientific history including Nobel prizes in cellular physiology beginning from ED Adrian and AV Hill.

Roger also inherited Richard Adrian's scientific style of an intensive benchside engagement and close interactions with relatively few colleagues of exceptional calibre in a consequently small research group.

They shared an awareness that results of lasting value depended not only on broad driving ideas, but also the detailed laboratory innovation needed to convert imagination into novel scientific truth, aptitudes and turns of mind relatively unfashionable in a hectic modern scientific age. Supervisor and student subsequently maintained a mutual loyalty: over subsequent years Roger kept contact with his supervisor to the end of the latter's life. In any case, Roger found little attraction in then available electrophysiological methods of recording extracellular response patterns in individual neurones within the enormous central nervous system population involved even in responses to simple sensory stimuli. Roger also had stimulating and productive conversations with Richard Adrian on Laplace transform and cable theory in the analysis of electric current flow in excitable cells. The former related to determinations of effective capacitance in distributed membrane networks (Adrian & Almers, 1973); the latter bore on novel voltage clamp methods applied to infinite cables (Adrian & Marshall, 1977). Roger also closely interacted with chemists like Gerry Smith in the Biochemistry Department, Ian Baxter and Jeremy Sanders in the University Chemical Laboratories, and John Kimura and colleagues in Denis Haydon's group in the Physiological Laboratory.

Although not a natural extrovert, Roger also developed a network of positive informal friendships and scientific liaisons that proved long-lasting. I first encountered Roger joining Richard Adrian's laboratory as his successor as PhD student as he commenced his research fellowship, and both of us were then at Gonville and Caius College. He then guided me in preparing physiological solutions for studies on membrane capacitative currents, giving me my first experience using Quikfit glassware. We continued to interact cordially and productively during his research fellowship whilst I had joined the faculty, when he was a sympathetic listener whilst I was completing my theoretical analysis of those results (Duane & Huang, 1982). These encounters often took place in laboratory corridors and stairwells in the evenings during his forays, calculator in his hip pocket, cycling between experiments in the Physiological and Chemical Laboratories at opposite ends of Tennis Court Road. We also regularly met on his visits to the United Kingdom after he returned to the United States, often when he visited Richard and Lucy Adrian. His formidable musical talent on the piano also made friends through musical soirees with Arieh Lew and Anne (Mrs. Richard) Keynes. I first met his then future wife Wendy Globe in the West Road Concert Hall in Cambridge at a chamber music concert.

Doctoral and postdoctoral studies in Cambridge: Biophysical methods for monitoring cell physiological activity

Roger's PhD thesis, awarded in 1977, on "The Design and Use of Organic Chemical Tools in Cellular Physiology", won the prestigious Gedge Prize in Biology in Cambridge, and award of a Comyns Berkeley Research Fellowship by Gonville and Caius College. Elections to such College research fellowships are highly competitive with independent scrutiny of the candidates' submitted works by distinguished outside

assessors, but then provided support for a free choice of research activities in Cambridge over a 5 year period, ideal for someone of Roger's independence and originality. This period likely established the foundations for Roger's scientific life. He began his developments of chemical, and later, molecular biological, probes that could be introduced into, or expressed, in the cell interior for studies of its physiological processes. This highly individualistic and elegant pursuit by someone extraordinarily gifted in organic chemistry initially focused on intracellular Ca^{2+} measurements, though later studies were inclusive of a wide range of further ions and molecules. This initial interest was timely: over the 1970s it became appreciated that cytosolic [Ca^{2+}] was a key central second messenger. Clarification of its modification following a triggering activation or regulatory step, and modulation of this process by exchanges between free and bound cytosolic compartments, intracellular Ca^{2+} stores and the extracellular space was central to understanding the regulation of cellular activity.

Development of electrode measurements for Ca^{2+} detection and quantification

This quest began with the most direct measurements obtained through accessing the intracellular space of excitable cells by intracellular electrodes, first used to determine membrane potentials (Adrian, 1956). Na^+, K^+, H^+ and Cl^--selective electrodes had already been available and widely used (Thomas, 1978) but use of Ca^{2+}-sensitive electrodes entailed further specific problems. There was the requirement for high selectivities of the electrode for Ca^{2+} over other potentially confounding cations, particularly Mg^{2+}, Na^+ and H^+, and high stability with minimal hysteretic responses with changing [Ca^{2+}]. Roger introduced more Ca^{2+}-selective, neutral, as opposed to organophosphate anion, ligands as liquid sensors (6). In addition sufficiently small (~0.4 μm) electrode tip diameters (4) were required to avoid penetration damage that in turn could artefactually elevate local [Ca^{2+}]. However, the high associated electrode resistances, of 20-30 GΩ and 60-120 GΩ in a solution of pCa=3 arising from 1.5 or 0.5 μm tip diameters required in turn entailed bespoke adaptations of electrometers of very high input impedance and small and stable bias currents (<10 fA) to avoid spurious signals.

The optimised electrodes yielded the theoretically expected Nernst relationships between voltage readout following subtraction of simultaneously recorded membrane potentials down to 1 μM free [Ca^{2+}] in 0.1 M KCl, with useful responses continuing to below 100 nM [Ca^{2+}] (Fig. 1). The free [Ca^{2+}] measurements agreed with established luminescence determinations in barnacle giant muscle fibres (1) and frog skeletal muscle (Blinks *et al.*, 1978). The latter used the fluorophore aqueorin, which contains three Ca^{2+} binding EF hand motifs, derived from the jellyfish, *Aequorea victoria,* found in the Pacific ocean off the North American west coast, which emits a fluorescent flash when agitated (Shimomura *et al.*, 1962; Shimomura, 1995). This jellyfish species was to become instrumental again later in Roger's scientific life (see below).

The electrode methods then proved applicable to ferret ventricular myocardium (2), and early *Xenopus laevis* embryos (3).

Optical techniques for Ca^{2+} measurement using bespoke organic molecules

Electrode methods thus permitted quantitative assessment of a wide range of [Ca^{2+}], were selective against Mg^{2+} and H^{+}, and amenable to incorporation into electrophysiological techniques. However, clarifying the cell physiological role of Ca^{2+} required monitoring techniques applicable to wide ranges of experimental conditions in a similarly wide range of cell types. The then available fluorescence techniques commended optical methods to this end. Optical methods show better Na^{+} rejection and faster responses in contrast to the slow response times of electrode methods, suitable for monitoring biological events. In contrast to monitoring conditions at a point, optical methods also provided [Ca^{2+}] estimates averaged over defined regions of interest and could also characterise spatial [Ca^{2+}] distribution with the subsequently available confocal microscopic techniques.

Roger's efforts, some begun with Timothy Rink, and Tulio Pozzan with whom he would have a prolonged scientific association, contributed much to optical measuring methods becoming the major current approach to studying cellular Ca^{2+} physiology. Such probes needed to show detectable alterations in emission or absorbance properties under excitation conditions compatible with recording and cell survival, the required Ca^{2+} specificity and quantifiable means for both transient and steady state [Ca^{2+}] measurements. Aequorin luminescence signals offered reproducible representations of Ca^{2+} transients but had low Ca^{2+} affinities and a 2.5 power relation between signal and [Ca^{2+}] complicating quantitative interpretation particularly with heterogenous myoplasmic ion distributions (Blinks *et al.*, 1978). Subsequently available absorbance dyes showed higher and linear Ca^{2+} affinities and more rapid Ca^{2+} association (Antipyrylazo-III, <<~1 ms; dichlorophosphonazo-III, <2 ms; arsenazo-III, 2-3 ms) capable of tracking even rapid [Ca^{2+}] changes in skeletal muscle (Csernoch *et al.*, 1988). However, exemplars such as arsenazo-III and antipyrylazo-III show varied Ca^{2+} binding stoichiometries, forming 1:2 and 2:1 complexes to extents depending on dye concentration, and large absorbance changes in response to Mg^{2+} and H^{+} in addition to Ca^{2+} (Baylor, 2011). Furthermore, the presence of significant indicator concentrations could themselves perturb the systems under study: in skeletal muscle, antipyrylazo III and arsenazo III showed differing absorbance timecourses following large prolonged pulses to -20 mV suggesting that one or both dyes themselves influenced the Ca^{2+} transient (Palade & Vergara, 1982). Dye could also potentially bind to cytoplasmic components, or partition into noncytosolic compartments potentially confounding interpretations based on in vitro cuvette calibrations. Finally, all of these indicators required injection into cells, in common with micro-electrode Ca^{2+} measurements. This would have restricted study to robust and well-anchored individual large cells such as muscle fibres, squid giant axons and *Limulus* retinal cells.

An opportunity to provide Ca^{2+}-specific binding over physiologically useful [Ca^{2+}] arose from the cage formed by the four carboxyl groups in the established Ca^{2+} chelator ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)(Fig. 2a) giving 1:1 dye:Ca^{2+} binding (5). This began the rational development of high-affinity buffers and optical Ca^{2+} indicators beginning with replacing the methylenes linking oxygen and nitrogen by phenyl groups. In the analog 1,2-bis (2-aminophenoxy)ethane-*N,N,N0,N0*-tetraacetic acid (BAPTA), two benzene rings replace the methylene groups connecting the N to O atoms whilst conserving overall molecular geometry and Ca^{2+} specificity and binding (Fig. 2b). BAPTA is a widely used and effective intracellular Ca^{2+} buffer, with a Ca^{2+} binding less affected by pH, more selective for Ca^{2+} relative to Mg^{2+} and with faster forward and reverse binding rates than EGTA.

However, EGTA has a far ultraviolet absorption spectrum and does not fluoresce. BAPTA has an ultraviolet spectrum that alters with Ca^{2+} binding with a ~250 nm maximum excitation not ideal for fluorescence measurements. However, substitution of one of the oxy-benzenes with a methoxyquinoline ring in the novel compound quin-2 gave excitation and emission wavelengths of 340 nm and 492 nm respectively (Fig. 2c). The latter were unchanged with nevertheless sixfold greater fluorescence intensities with Ca^{2+} binding. [Ca^{2+}] measurements were calibratable at typical resting cytosolic $[Ca^{2+}]_i$ ($\sim 10^{-7} M$) or below through to 10^{-8}M. Other then available techniques showed detection optima only at activated [Ca^{2+}] ($\sim 10^{-6} M$) and signals below detection limits at resting [Ca^{2+}].

Finally, cell incubation in solutions containing the membrane-permeant acetomethoxy (AM) ester quin-2-AM accomplished atraumatic yet consistent intracellular access without micromanipulation or plasma membrane disruption. Endogenous esterases then split off the ester groups releasing and trapping the membrane-impermeant quin-2 tetra-anion (7) (Fig. 2d). Increases in quin-2 fluorescence with increased [Ca^{2+}] could be monitored by conventional cuvette spectrofluorimeter. Quin-2 thus became strategic in cytosolic [Ca^{2+}] measurements in a wide range of mammalian cells and cell suspensions including lymphocytes, thrombocytes, sperm, neutrophils and macrophages, particularly in assessments of the roles of Ca^{2+} in stimulus-response coupling (8, 9). In future years this approach to cellular introduction of esterified membrane permeant derivatives was extended to studies of the roles of candidate cell messenger molecules, such as phosphatidylinositol 3,4,5-trisphosphate, in cell physiology (25).

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At the University of California at Berkeley: new dyes measuring and manipulating intracellular [Ca^{2+}] and other ions

After a significant search, likely reflecting employment and opportunity difficulties experienced by individuals pursuing interdisciplinary research directions in the United Kingdom, Roger accepted

appointment as assistant professor in the Department of Physiology and Anatomy in the University of California, Berkeley then headed by Terry Machen in 1981, where he was to remain 8 years. He here developed further optimised Ca^{2+}-sensitive dyes in collaborations with Stephen Adam and Robert Zucker, often extending to commercial application and distribution, of the consequent agents now in widespread use in cellular physiology. All these were loaded through incubation with their AM derivatives. The compounds combining an 8-coordinate tetracarboxylate chelating site with stilbene chromophores gave improved Ca^{2+}-selectivity over other divalent cations and a slightly lower Ca^{2+}-affinity than quin-2 (5). These Ca^{2+} affinities together ranged from K_d = 110 nM (quin-2) to 90 μM (Fluo-5N) covering typical physiological $[Ca^{2+}]$ in a wide range of cell types through their quiescent and active states. Their longer excitation wavelengths compared to quin-2 (339 nm) avoided requirements for ultraviolet irradiation potentially exciting cell autofluorescence and causing cell damage. Incorporating the stilebene ethylenic linkage into a heterocyclic ring enhanced quantum efficiency and photochemical stability giving ≤30-fold increased fluorescence signals. This improvement in extinction coefficient and fluorescence quantum yield over that of quin-2 (<5000 and 0.03 to 0.14 respectively) also reduced the required cell loading (mM in the case of quin-2) that could potentially lead to the dye itself buffering intracellular $[Ca^{2+}]$ and perturbing the physiological processes investigated.

Finally, the new dyes showed wavelength in addition to intensity changes upon Ca^{2+} binding, whereas quin-2 signals $[Ca^{2+}]$ only through fluorescence intensity giving readouts sensitive to experimental variations in illumination intensity, emission detection, dye concentration, and effective cell thickness in the optical beam. Measurements using spectral shifts in excitation and/or emission with Ca^{2+} binding circumvented these sources of artefact (11). Thus, Indo-1 has a dual emissions peak, with its main emission shifting from 475 to 400 nm with Ca^{2+} binding. It has found extensive use in flow cytometry (Fig. 3a). Fura-2 emits at a single 510 nm wavelength but its peak excitation shifts from ~380 to 350 nm with Ca^{2+} binding giving emission ratios at these excitation wavelengths directly related to $[Ca^{2+}]$. It has found wide cell physiological use. Its high photon yield has made it useful even for real time, video rate, measurements of local intracellular $[Ca^{2+}]$ (Cannell *et al.*, 1987) (Fig. 3b).

These accomplishments began the creation of a wide range of novel molecular probes detecting and monitoring multifarious ions and molecules in their associated physiological processes (Haugland, 2010). First, these new variants permitted $[Ca^{2+}]$ measurements under differing conditions and cell types. Fluo-3 is amenable to visible, including 488 nm argon laser excitation, increasing its fluorescence with Ca^{2+} binding about a single peak 525 nm emission wavelength, close to that detected in fluorescein isothiocyanate (FITC) measurements (Fig. 3c). Its more rapid Ca^{2+} dissociation compared to that of fura-2 permitted tracking of rapid $[Ca^{2+}]$ kinetics in skeletal and cardiac muscle. This was particularly useful in confocal laser scanning

microscope detection of microscopic Ca^{2+} release events ("calcium sparks") (Cheng *et al.*, 1996), as well as the resulting subliminal propagated Ca^{2+} waves with reflecting increased sarcoplasmic reticular ryanodine receptor Ca^{2+} release channel activity, following uncoupling from its surface dihydropyridine receptor in skeletal (Chawla *et al.*, 2001) or pro-arrhythmic circumstances in cardiac muscle (Hothi *et al.*, 2008; Goddard *et al.*, 2008).

Second, it became possible to detect and measure concentrations of entities other than intracellular Ca^{2+}. For example, the carboxyfluorescein derivative 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) utilised its carboxyl- groups in H^+ rather than Ca^{2+} determination. BCECF was made available with a cell-permeant AM analog, and had single emission (535 nM) and dual (~490 and ~440 nm) wavelengths. Its pKa (~6.98) and linear responses between pH 6.4-7.4 proved applicable in cell physiological applications in gastric oxyntic cells (10).

Third, light could conversely release ('uncage') Ca^{2+} from Nitr-2, and the later developed Nitr-5 and DM-nitrophen (Zucker, 1992). This comprised the Ca^{2+}-chelating BAPTA linked to a nitropiperonyl group photolysable by near ultraviolet (300-400 nm) light. The latter markedly reduces its Ca^{2+} dissociation constant from 160 and 630 nM to 7 and 18 μM at 0.1 and 0.3 M ionic strengths respectively, uncaging its bound Ca^{2+} thereby substantially altering intracellular [Ca^{2+}] (13) (Fig. 4a-c), a property used to good effect in experiments photochemically manipulating neuronal ion currents (14). Roger was subsequently to further develop his photolytic release approach to other bioactive messengers further resolved spatially in three dimensions to the focus point of the exciting laser source. Thus, conjugating brominated 7-hydroxycoumarin-4-ylmethyls to the candidate messengers enhanced uncaging yields with two-photon, infra-red as opposed to ultraviolet excitation, and permitted the first three dimensional mapping of neuronal glutamate sensitivity in rat cortical brain slices (26).

Development of fluorescent protein markers

Even before his move to San Diego in 1989, work done by Alexander Glazer on fluorescent phycobiliproteins, prompted Roger to begin interests in the fluorescent imaging of cellular cyclic 3',5' adenosine monophosphate (cAMP). He sought as fluorescence detection probes natural cAMP-binding proteins thereby ensuring the required intracellular cAMP binding affinities and selectivities. In the absence of cAMP, phosphokinase A (PKA) is inactive with tightly associated regulatory and catalytic subunits (Fig. 5). cAMP binding to the regulatory subunits dissociates and activates the catalytic subunits to transfer phosphate groups from ATP onto particular proteins. Making the enzyme optically signal for such cAMP-binding events returned Roger to his graduate interest in fluorescence resonance energy transfer (FRET) between two light-sensitive chromophores sterically close to each other. An excited donor chromophore

might then transfer energy to the acceptor by nonradiative dipole–dipole coupling. This situation was created by attaching one fluorophore type to the regulatory and another type to the catalytic subunits. FRET could then take place involving regulatory and catalytic subunits in close contact in the intact PKA, but not following the dissociation produced by cAMP binding. The two situations would then result in differing fluorescence wavelengths. These experiments, conducted with Susan Taylor's group involved much persistent work needing large quantities of recombinant PKA subunits but emerged with viable methods to combine fluorescein-labeled catalytic subunits with rhodamine-labeled regulatory subunits to produce FRET-based sensors for cAMP (15). This was to find applications in studying osteoblastic (18), melanocyte (16) and *Aplysia* neuronal cell types (17).

At the University of California at San Diego: development of genetically encoded macromolecular indicators

Use of fluorescent proteins thus constituted an elegant extension of the small-molecule dyes. However, the necessary proteins then had to be expressed and purified in large quantities for bespoke attachment of two different dyes *in vitro* to distinct protein domains or subunits whilst preserving protein function. As in the early work, the protein would also have to be injected. These issues led to explorations of genetically encoded macromolecular indicators involving introduction of genes that would encode the two fluorescent proteins of the appropriate colours, into the cell types under study. This would involve less demanding and established procedures for cell transfection of much smaller quantities of DNA than of protein molecules followed by a selective propagation of the cells in which this transfection was successful. This quest returned Roger to the jellyfish *Aequorea victoria,* the source of aqueorin (Shimomura *et al.*, 1962) so useful in the classical physiological studies. Shimomora had found, extracted, purified green fluorescent protein (GFP) from about 10,000 specimens, characterising its physicochemical, including its excitation and emission fluorescence spectral properties under various conditions. He identified GFP was a FRET acceptor from an aequorin donor, thereby giving a in vivo green fluorescence as opposed to the blue fluorescence shown by purified aequorin under ultraviolet excitation (Morise *et al.*, 1974), and identified its *p*-hydroxybenzylideneimidazolinone chromophore within the GFP peptide chain (Shimomura, 1979).

A characterisation of part of the GFP gene by Douglas Prasher (Prasher *et al.*, 1992) then initiated an interlocking group of studies in different laboratories. Roger worked on generation and fluorescence properties of GFP using *S. cerevisiae* with Roger Heim and Scott Emr. Martin Chalfie at Columbia who had first demonstrated ultraviolet-induced fluorescence in GFP injected into invertebrate cells suggesting potential uses as a biological marker, worked on its expression in *Escherichia coli* and *Caenorhabditis elegans*. Roger's studies yielded a Y66H mutant (BFP) with improved and consistent blue fluorescence signals relative to the original wild-type GFP. Further protein modifications that stereochemically

accommodated the tryptophan yielded a Y66W mutant encoding a cyan fluorescent protein (CFP) (19, 21) (Fig. 6A). In a FRET pair, protein conformational changes in an ultraviolet-excited BFP induce transfer of fluorescence energy to the GFP (Fig. 6B). This would require the acceptor GFP to be excitable by the blue wavelengths emitted by the donor BFP. However, the latter has a large ultraviolet and small blue excitation peak. Nevertheless, the unwanted UV peak disappeared and blue peak increased 5–6 fold with further +10 nm wavelength shifts with a S65T mutation (21). Proof-of-principle experiments then introduced peptide links between the BFP and the GFP-S65T, or other mutants with GFP-S56T-like excitation spectra. Selective ultraviolet excitation of BFP then indeed gave distinct green and blue emissions in the presence and absence of FRET transfer before and after trypsin proteolysis of the peptide. Subsequent studies confirmed that S65T was an optimal fluorophore, adding a further F64L mutation permitting folding at higher temperatures (Cormack *et al.*, 1996). The double mutant, "enhanced (S65T- F64L) GFP", is the widely used variant marketed by Clontech.

Structural determinations for the GFP-S65T revealed a cylindrical structure, diameter 2.4 nm, length 4.0 nm, comprising eleven β-strands surrounding an axial helix into which the chromophore was inserted at the molecule centre (22) (Fig. 7). It was thus shielded from both solvent and extraneous enzyme action but within a cavity suggesting room for a π-stacked aromatic ring adjacent the chromophore; this was tested by a T203Y (and other) mutation. The latter red-shifted both excitation and emission maxima by ~20 nm. The resulting yellowish YFP and its subsequent mutational variants proved good FRET acceptors from CFP giving a CFP/YFP FRET pair as an alternative to the BFP/GFP combination.

Roger was also to clarify the spontaneous formation of the *p*-hydroxybenzylideneimidazolidinone chromophore from the serine-65, tyrosine-66, and glycine-67 residues within GFP (Cody *et al.*, 1993). This involved the surprising formation of a de novo heterocyclic ring with dehydrogenation of the α-β C-C to a C=C. The latter required a hydrogen acceptor for which he implicated atmospheric O_2. Anaerobic cultures of the GFP-expressing bacteria failed to fluorescence despite the presence of synthesised protein. However, fluorescence appeared a few hours after re-exposure to air (19), a property to become useful in physiological studies.

Coupling the FRET donor and acceptor GFPs to detector proteins

Application of FRET-visualising specific intracellular signals next requires conjugation of the FRET components to a molecule specifically binding the entity being investigated. Roger's attempts to attach donor and acceptor FPs to opposite ends of the cytosolic domain of the recently cloned inositol 1,4,5-trisphosphate (InsP3) receptor with Atsushi Miyawaki likely reflected his interest in chemical signalling between cell membranes. This had led to explorations of the involvement of this important messenger in

skeletal muscle excitation-contraction coupling (12) and of Ca^{2+} influx factor (20) signalling from intracellular Ca^{2+} stores to surface membrane in store-operated Ca^{2+} entry (Putney, 2017). The former subsequently proved instead to involve direct rather than chemical coupling between surface L-type Ca^{2+} channels and intracellular sarcoplasmic reticular ryanodine Ca^{2+} release receptors (Huang *et al.*, 2011). However, difficulties arising from the still incomplete knowledge of the receptor's InsP3 binding returned Roger to further detectors for intracellular Ca^{2+} with Mitsuhiko Ikura. This involved attaching BFP, and subsequently CFP, to the N terminus of calmodulin (CaM). Conversely, S65T, and subsequently YFP was attached to the C-terminus of its M13 target peptide. The resulting structure fused these CaM and M13 entities (24, 27).

These resulting genetic-encodable probes ('cameleons'), amenable to long term chronic use applicable to any targeted cell or organism into which their DNA could be introduced, provided amongst the most popular approaches to imaging activity in identified neurones extending to intact nervous systems. They also led to broadening of this approach to other biologically important molecules. Significant persistence led to linkers allowing fusion of the fluorescent proteins to PKA whilst preserving the ability of PKA subunits to respond to cAMP. The resulting detectors could image subcellular compartmentation of cardiomyocyte cAMP following catecholaminergic stimulation (Zaccolo & Pozzan, 2002). Finally, a modified cameleon pattern, replacing M13 by a kinase peptide substrate and CaM by a protein domain containing a phosphoamino acid binding domain that binds phosphorylated Ser, Thr, or Tyr, made it possible to visualise activity in protein kinases specific for phosphorylating the respective Ser, Thr or Tyr. Kinase phosphorylation of the target residue results in its being complexed altering the distance or orientation between donor and acceptor fluorescent proteins (Zhang & Allen, 2007), further illustrating this new and soon-indispensable research tool.

Completing the fluorescent protein colour palette; extension of FRET to detecting membrane phenomena

Further developments yielded a fuller repertoire of fluorescent proteins covering a wide optical spectrum with further photoswitching capacities (Shaner *et al.*, 2007) (29, 34). Discovery and availability of a gene encoding a red coral fluorescent protein (DsRed) (Matz *et al.*, 1999) prompted Roger to broaden his fluorescent protein explorations beyond GFP. DsRed is an obligate tetramer whose chromophore begins similarly as that of GFP. However, a further dehydrogenation yields an acylimine, stable only when embedded within the intact protein then showing red-shifted excitation and emission spectra (Wall *et al.*, 2000; Yarbrough *et al.*, 2001). A sequence of directed evolution generated a monomeric red fluorescent protein (RFP), more amenable to reliable protein fusions than the tetramer, and which generated an

extensive set of monomeric fluorescent proteins giving a palette of emission maxima covering the rest of the visible spectrum out to 648 nm (28).

Roger also turned his hand to manipulating the FRET technique itself in a return to early interests during his PhD in optical measurements of cell membrane potentials. This improved established but often relatively slow or insensitive one-fluorophore indicator methods. A two-component FRET-derived signalling employed as FRET donors fluorescent lectins, and later, a coumarin-labelled phosphatidylethanolamine phospholipid bound on one side of the plasma membrane (Fig. 8). These transferred fluorescence resonance energy to a highly fluorescent charged intramembrane bis(1,3-dihexyl-2-thiobarbiturate)tri-(or penta)methineoxonol acceptor when this electrophoresed from the inner to the outer membrane face following membrane potential change, giving unprecedented large sensitivities (>50% increased fluorescence ratio per 100 mV) and rapid (<0.4 ms) time constants amongst optical indicators (23).

From cellular to systems physiology, clinical translation and distribution of novel agents

Towards the end of his scientific life, Roger added optical detection tools particularly applicable to the whole organ or animal as opposed to the single cell level, with potential clinical, neurosurgical, cardiovascular or oncological applicability, some of these in collaboration with Quyen Nguyen. A novel fluorescently labeled injectable nerve binding probe F-NP41 directed towards basement membrane laminin may improve intraoperative visualization of chronically transected nerves (31, 33) potentially facilitating development and execution of surgical nerve repair procedures (Glasby *et al.*, 1986; Gattuso *et al.*, 1988). A positron emission tomographic probe using injected erythrocytes labelled with a positron-emitting, fluorescent multimodal imaging probe suggesting a means for detecting intracranial haemorrhage as an alternative to 99mTc-labelled agents (36) that could potentially also be useful in experimental magnetic resonance imaging studies of cerebral pathophysiological processes (Smith *et al.*, 2006). His introduction of cell-penetrating peptides showing measurable ratiometric fluorescence changes following intracellular cleavage by tumour-associated proteases could contribute to early diagnosis of malignant lesions (35). Both such activatable cell-penetrating peptides and their Gd-loaded dendrimeric forms proved also selectively to accumulate in tumour cells, the latter enhancing their detection and suggesting a possible future means for their diagnosis under magnetic resonance imaging (32). Activatable cell-penetrating peptides were similarly used to target the anti-tubulin radiosensitizer monomethyl auristatin E specifically to tumour cells in explorations of applications with therapeutic, in addition to diagnostic potential (30).

Throughout his career, Roger sought to make his novel agents available to colleagues. In his early development of quin-2, the UK National Research and Development Corporation (NRDC) failed to be convinced of the commercial relevance of a generalisable structure sensing Ca^{2+} with unprecedented

accuracy and selectivivity. Demand for quin-2 and its acetomethoxy derivative was then met by Lancaster Synthesis, now part of the Johnson Matthey Company Alfa Aesar. Roger's activities were subsequently to result in ~160 United States patents. He also founded companies, such as those with Charles Zuker: The Aurora Biosciences Corporation producing drug discovery tools using fluorescent markers and Senomyx seeking taste receptor modulators. His patents also prompted foundation companies by others, such as Molecular Probes.

'The road less travelled by…'

In 2014, Roger suffered a haemorrhagic stroke, impairing his mobility, but certainly not his intellect when I met him for the last time in November 2015 marking his election as Honorary Fellow of the Cambridge Philosophical Society. He then delivered a lecture on possible roles of stable proteins close to synaptic contacts at dendritic spines in memory formation, then his current interest. His passing, aged 64, in 2016, while cycling outdoors, in Eugene, Oregon, to where he had moved with Wendy from San Diego, left his wife, Wendy, his stepson, Max Rink, and his brothers, Richard and Louis. It tragically ended a unique and brilliant career. In a 'road less travelled by' (Frost, 1916), this was devoted to applying his exceptional chemical talents to the development of diverse methods measuring key ions and molecules in cell physiology. These in turn provided tools to permit important physiological discoveries and determination of their mechanisms even during his own lifetime. He also left a large complement of graduate students and postdoctoral fellows grateful for his mentoring. Science also lost an eloquent and inspiring communicator and lecturer.

He bequeathed to the scientific community an extensive range of fluorescent small molecule and protein probes that have already and will continue to provide important means of directly visualising a wide range of key biochemical processes within living cells and organisms. These agents could be variously introduced as permeant molecules or through their intracellular gene expression brought about by molecular biological procedures. They variously acted as involving single or interacting (FRET) fluorescence components. This gave detection techniques relevant to the entire range of cell physiological processes beginning with whether their underlying host genes are switched on or off, the generation, translocation and interactions involving their protein products, as well as the physiological processes that result from their actions. Their readouts took the form of aesthetically satisfying spectral signals echoing his enthusiasm for amateur photography and treks in the wild outdoors, and reminiscent of Paul Gauguin's tributes to one of his own forebears: "It is striking that Delacroix, so preoccupied with colour, reasoned it equally a law of physics and the imitation of nature. Colour! This language so profound, so mysterious, the language of the dream."(Gauguin, 1896).

Most significant honours and awards

Roger was co-winner of the 2008 Nobel Prize in Chemistry with Osamu Shimomura, of the Woods Hole Marine Biological Laboratory and Martin Chalfie at Columbia University, USA. The Nobel citation particularly mentioned his contributions to our understanding of the fluorescence properties of GFP and related proteins, leading to their applications in studies of dynamic processes in living systems. In his typical fair minded way, he arranged for Douglas Prasher who began this quest in studying GFP, to be present at the ceremony. In addition to the awards cited above, he was also recipient of the Lamport Prize of the New York Academy of Sciences (1986), A Javits Neuroscience Investigator award (NINCDS, 1989-96), the Gairdner Foundation International Award (1995), the Artois-Baillet-Latour Health Prize (Belgium, 1995), the American Chemical Society Award for Creative Invention (2002), the HP Heineken Prize for Biochemistry and Biophysics, the Royal Netherlands Academy of Sciences (2002), the Max Delbruck Medal in Molecular Medicine, the Max Delbruck Institute, Berlin (2002), the Wolf Prize, Israel (2004) and the Keio Medical Science Prize, Keio University, Japan (2004) and the Lewis S Rosenstiel Award for distinguished work in basic biomedical science (2006), amongst others. He was elected member of the US Institute of Medicine (1995), the American Academy of Arts and Sciences (1998), the U.S. National Academy of Sciences (1998) and foreign member of the Royal Society of London (2006), and to an honorary fellowship of the Cambridge Philosophical Society (2014).

Acknowledgements

Photograph of Roger Tsien: Michael Spencer, NIH Record, volume LXII no 7, page 1, 2 April 2010, reprinted by permission, National Institutes of Health, Bethesda Md., USA. Figure 7 reproduced by permission from Fig. 1 of Brejc K, Sixma TK, Kitts P a, Kain SR, Tsien RY, Ormo M & Remington SJ (1997). Structural basis for dual excitation and photoisomerization of the Aequorea victoria green fluorescent protein. Proc Natl Acad Sci 94, 2306–2311. Copyright (1997) National Academy of Sciences.

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Figure captions

Figure 1. Development and use of Ca^{2+} sensitive electrodes. (a) Potential difference, E_{Ca2+} developed across Ca^{2+}-selective membrane relative to reference potential V_0 as a result of Ca^{2+} activities in the sampled fluid, a_s, and within the electrode a_f. (b) Microelectrode measurement configuration to measure Ca^{2+} activity in an excitable cell with membrane potential E_m, comparing the potentials generated by the tetraphenylphosphonium oxonol Ca^{2+} sensor microelectrode and voltage sensitive electrode impalements E_2 and E_1 respectively. (c) Expected calibration plots: the theoretical Nernst plot for Ca^{2+} in which a pCa of 3 is assigned zero voltage.

F*igure 2. Ca^{2+}-chelating compounds leading to development of quin-2.* (a) EGTA illustrating cage formed by the four carboxyl groups. The latter geometry is conserved in (b) the analog BAPTA despite replacements of the methylene groups. (c) The acetomethoxy (AM)- ester of the similarly Ca^{2+} binding indicator quin-2 readily permeates cell membranes where endogenous esterases cleave the ester groups yielding (d) quin-2 whose fluorescence increases sixfold with increased $[Ca^{2+}]$.

Figure 3. Compounds developed for intracellular $[Ca^{2+}]$ measurement ((a)-(c)) and its manipulation ((d) to (f)). (a-c) Optical dyes built on the 8-coordinate tetracarboxylate chelating site with stilbene chromophores marked out by dotted lines: (a) the emission dye indo 1; (b) the dual-excitation dye fura 2 and (c) single-excitation fluo-3. (d-f) 'Uncaging' of Ca^{2+} exemplified in Nitr-2 in which ultraviolet light converts (d) the 'caged' version to (e) its hemiketal, whose conversion to (f) the nitrosobenzophenone greatly increases the Ca^{2+} dissociation constant of the BAPTA entity.

Figure 3. Compounds developed for intracellular $[Ca^{2+}]$ measurement ((a)-(c)). Optical dyes built on the 8-coordinate tetracarboxylate chelating site with stilbene chromophores marked out by dotted lines: (a) the emission dye indo 1; (b) the dual-excitation dye fura 2 and (c) single-excitation fluo-3.

Figure 4. Ca^{2+} 'Uncaging' properties exemplified in Nitr-2. Ultraviolet light converts (a) the 'caged' version to (b) its hemiketal, whose conversion to (c) the nitrosobenzophenone greatly increases the Ca^{2+} dissociation constant of the BAPTA entity.

Figure 5. Fluorescence resonance energy transfer (FRET) applied to a phosphokinase A (PKA) cAMP probe. The fluorescein unit on the catalytic and the rhodamine unit on the regulatory subunit of PKA form a FRET pair before dissociation of the PKA units following cAMP binding which consequently alters the overall emission wavelength.

Figure 6. (A) Comparison of excitation and emission properties of genetic variants for GFP and (B) their assembly into fluorescence resonance energy transfer (FRET) pairs.

Figure 7. Ribbon diagram of WT GFP structure. Red: α-helices, green: β-strands; ball and stick model: chromophore. (Reproduced by permission from Fig. 1 of Brejc K, Sixma TK, Kitts P a, Kain SR, Tsien RY, Ormo M & Remington SJ (1997). Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. Proc Natl Acad Sci 94, 2306–2311. Copyright (1997) National Academy of Sciences.)

Figure 8. FRET applied to membrane potential detection. In the present example, impermeant donor fluorophore is located on the extracellular face of the plasma membrane, through conjugation with phospholipid. The charged intramembrane oxonol acceptor preferentially binds to the extracellular membrane side in fully polarised resting membrane, then coming into close proximity with the donor thereby permitting FRET. Depolarisation translocates the oxonol to the intracellular membrane side, increasing emission from the donor relative to that of the acceptor.

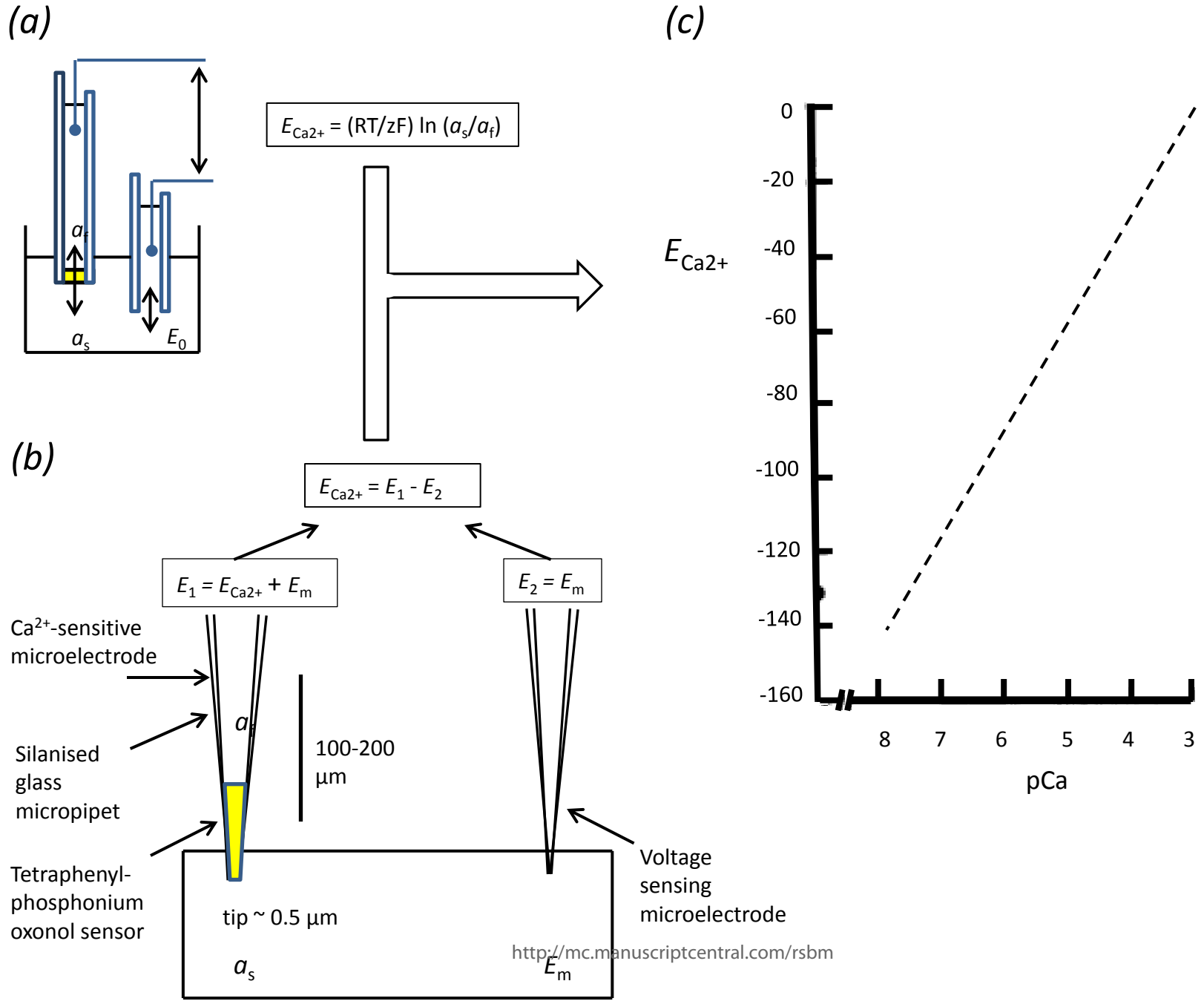
Author biography

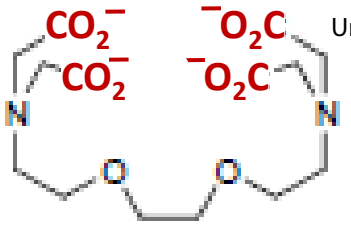
Christopher Huang (DM, DSc (Oxford), MD, ScD (Cambridge), FRSB, FESC) won a Florence Heale Open Scholarship to read Medicine and Physiology at The Queen's College, Oxford. He completed preregistration clinical appointments with Profs. Sir David Weatherall and John Ledingham in the Nuffield Department of Medicine. He then gained a Medical Research Council Scholarship to pursue membrane biophysics in the Physiological Laboratory and Gonville and Caius College, Cambridge, with Prof. Lord Richard Adrian as supervisor, directly following Roger Tsien. Following PhD completion eighteen months later, he became successively assistant and full University Lecturer in Physiology, Reader and finally Professor of Cell Physiology at Cambridge, whilst being Fellow and Director of Medical Studies at Murray Edwards College, and holding an honorary senior research fellowship in the Biochemistry Department. He investigates transduction of biological signalling events and their cellular and systems level propagation by

electrophysiological, optical confocal microscopic and mathematical modelling methods. He first analysed voltage- and ligand-coupled activation of intracellular Ca^{2+}-mediated physiological triggering by ryanodine receptors in striated muscle and osteoclast cells respectively, and its implications for cellular electrolyte homeostasis. He then pursued translational interests in nerve repair and experimental magnetic resonance imaging studies of cerebral cortical spreading depression in relation to migraine aura. His current work analyses cardiac arrhythmogenic mechanisms and means for their correction in murine genetic channelopathic and metabolic exemplars for clinical cardiovascular disease. He received the LEPRA (British Leprosy Relief Association), Benefactor's (Queen's College, Oxford) and Brian Johnson academic prizes (University of Oxford), and the Rolleston (University of Oxford) and Gedge Prizes (University of Cambridge) for physiological research. He is/has been editor of the Journal of Physiology, Monographs of the Physiological Society, Biological Reviews, BMC Physiology and Europace, and successively, Member of Council, Biological Secretary and President of the Cambridge Philosophical Society, and member of the British Heart Foundation Fellowships Committee and Advisory Council. He is/has been independent nonexecutive director of Hutchison China Meditech Ltd., developing novel cell signalling-targetted therapeutic oncological drugs, over the duration between its London Alternative Investment Market (AIM) and New York NASDAQ listings, and Hutchison Biofilm Medical Solutions Ltd., seeking biofilm-based antimicrobial agents, and is Manager of the Prince Philip Scholarship fund.

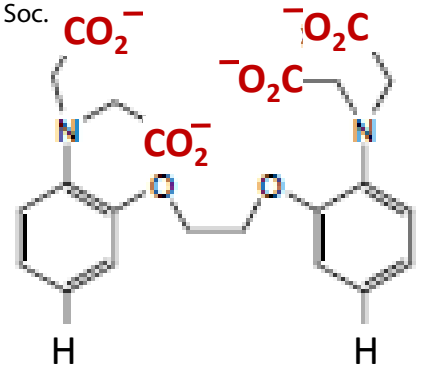
Frontispiece

Fig. 1

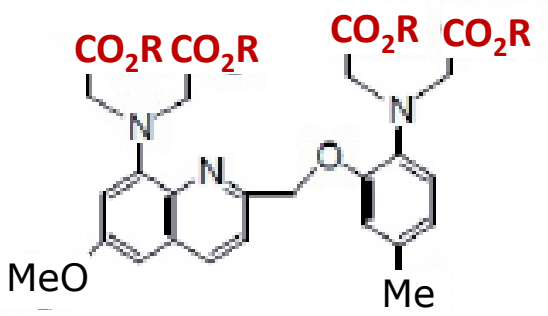

(a)
$E_{Ca2+} = (RT/zF) \ln (a_s/a_f)$
a_f
a_s
E_0
(b)
$E_{Ca2+} = E_1 - E_2$
$E_1 = E_{Ca2+} + E_m$
$E_2 = E_m$
Ca^{2+}-sensitive microelectrode
Silanised glass micropipet
Tetraphenyl-phosphonium oxonol sensor
a_f
100-200 µm
tip ~ 0.5 µm
a_s
E_m
Voltage sensing microelectrode
(c)
E_{Ca2+}
0
-20
-40
-60
-80
-100
-120
-140
-160
8
7
6
5
4
3
pCa



(a) EGTA



(b) BAPTA

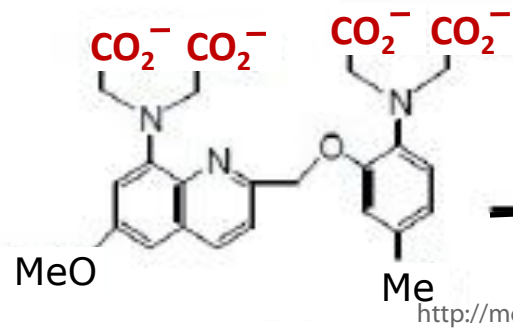


(c) Quin-2-AM

Me = CH_3 methyl-

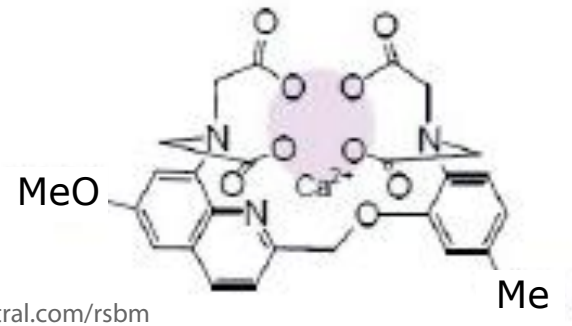
R = CH_3CO- acetomethoxy-

Intracellular
acetomethoxy- cleavage



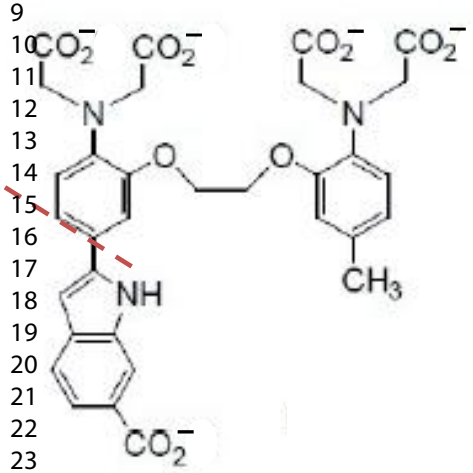
(d) Quin-2

Ca^{2+}

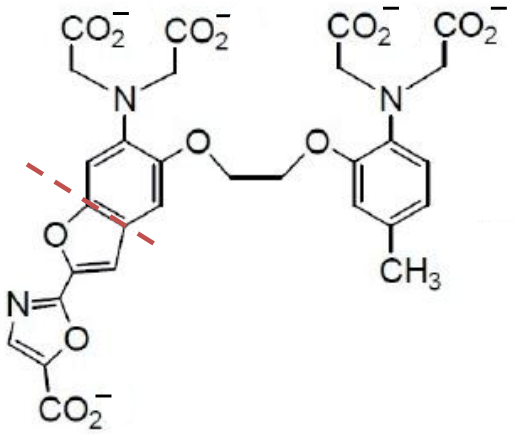


(e) Quin-2-Ca^{2+}

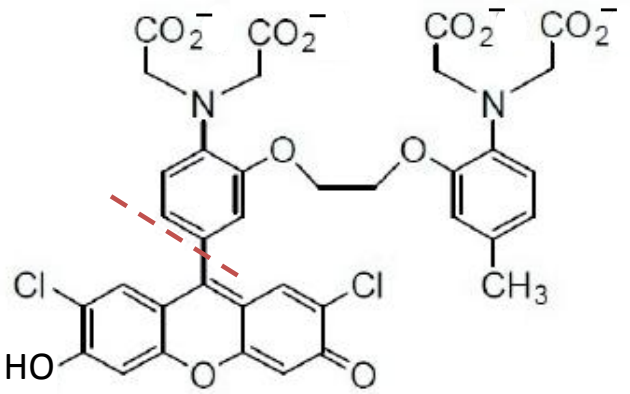
A



(a) Indo-1



(b) Fura-2

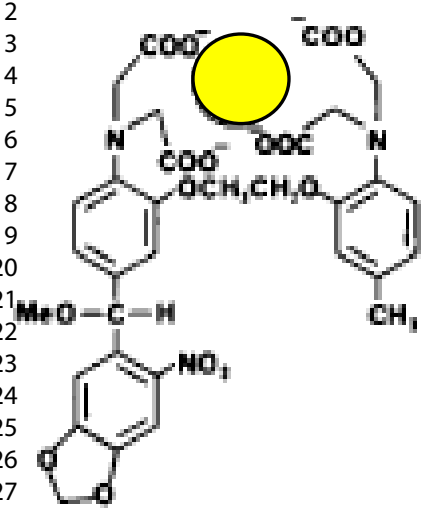


(c) Fluo-3

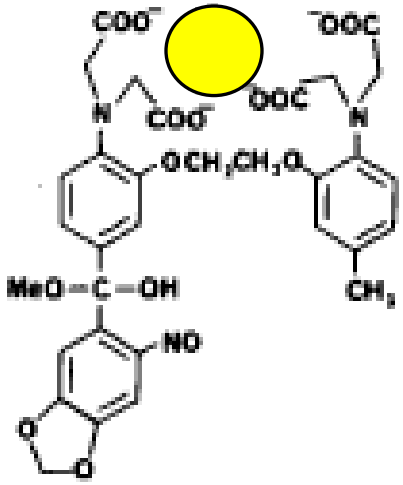
CO_2^-

Figure 4

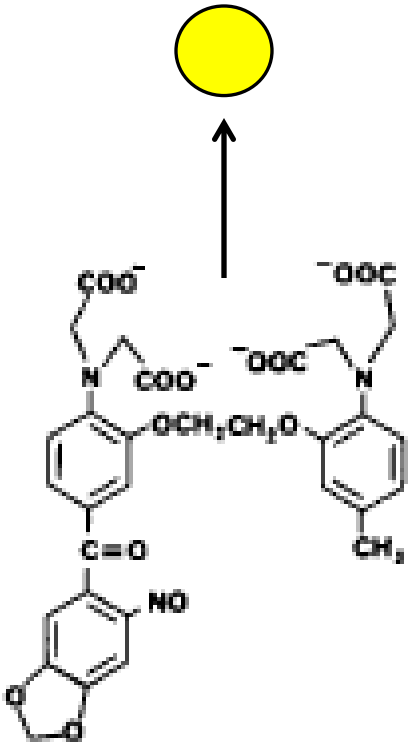
= Ca^{2+}



(a) Nitr-2 – Ca^{2+} complex

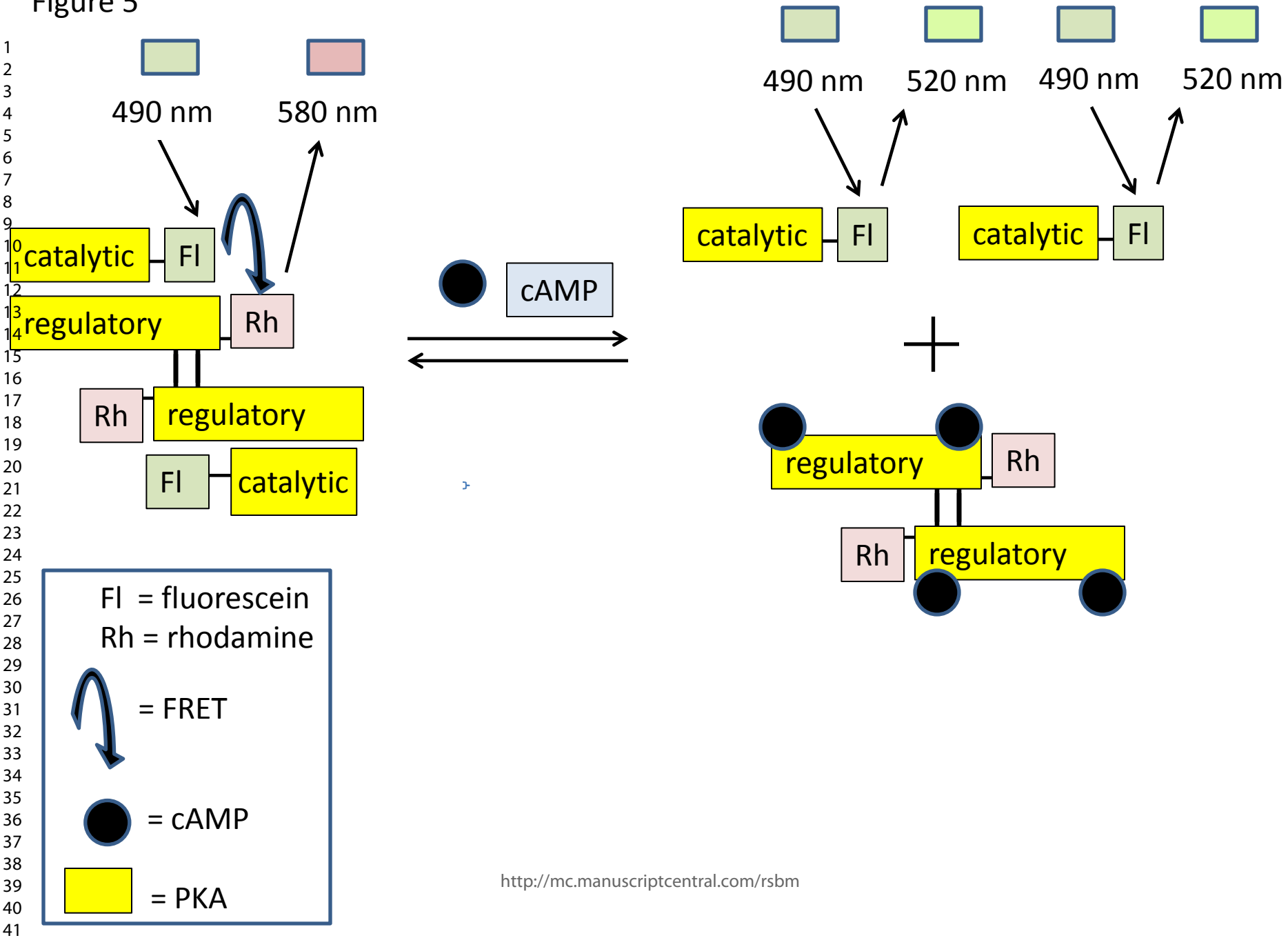


(b) Nitr-2 (hemiketal)



(c) Nitr-2 (nitrosobenzophenone)

Figure 5


490 nm
580 nm
catalytic
Fl
regulatory
Rh
Rh
regulatory
Fl
catalytic
cAMP
490 nm
520 nm
490 nm
520 nm
catalytic
Fl
catalytic
Fl
regulatory
Rh
Rh
regulatory
Fl = fluorescein
Rh = rhodamine
= FRET
= cAMP
= PKA

Under Review for Bio. Mems. & Sys.

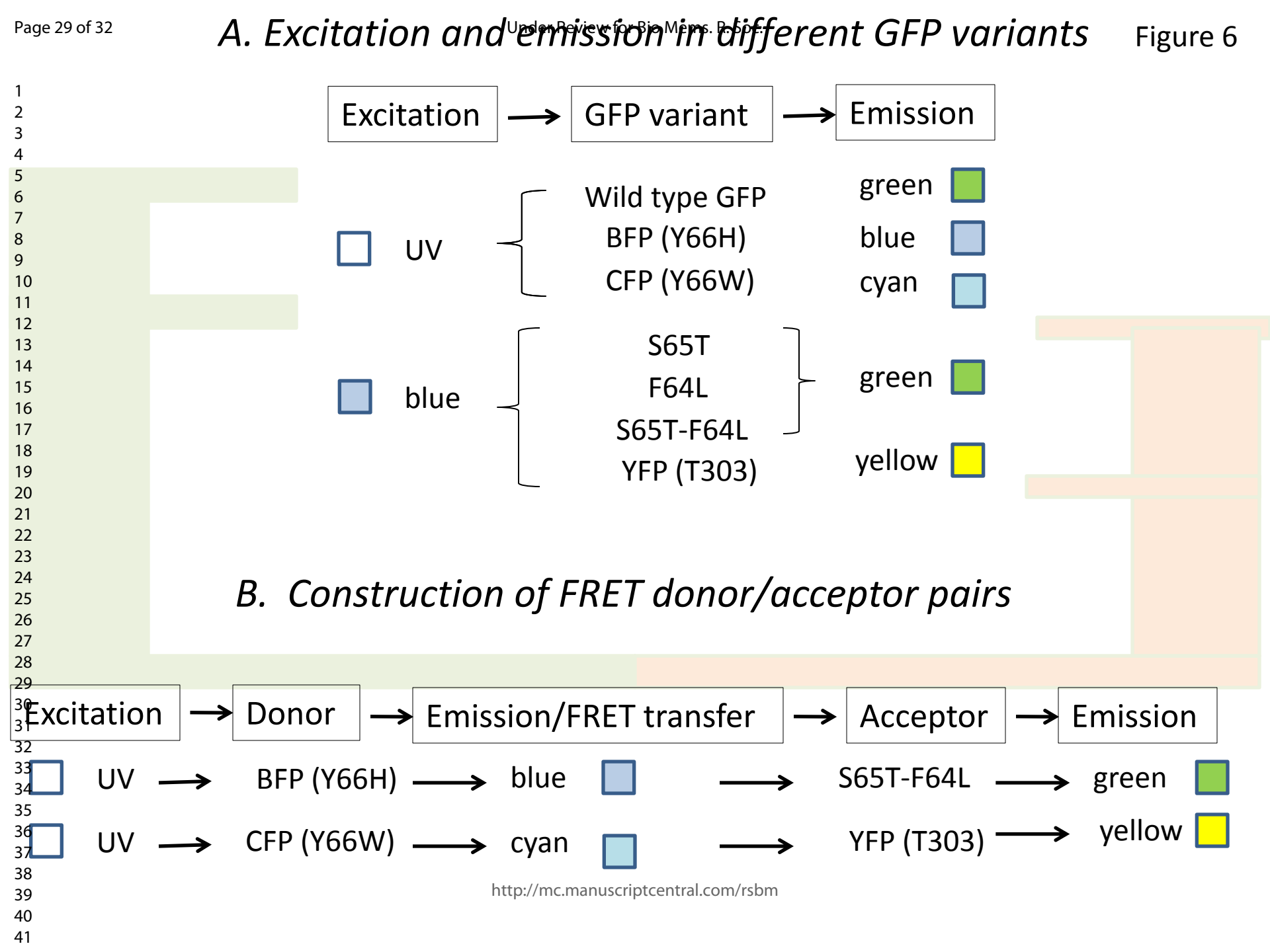
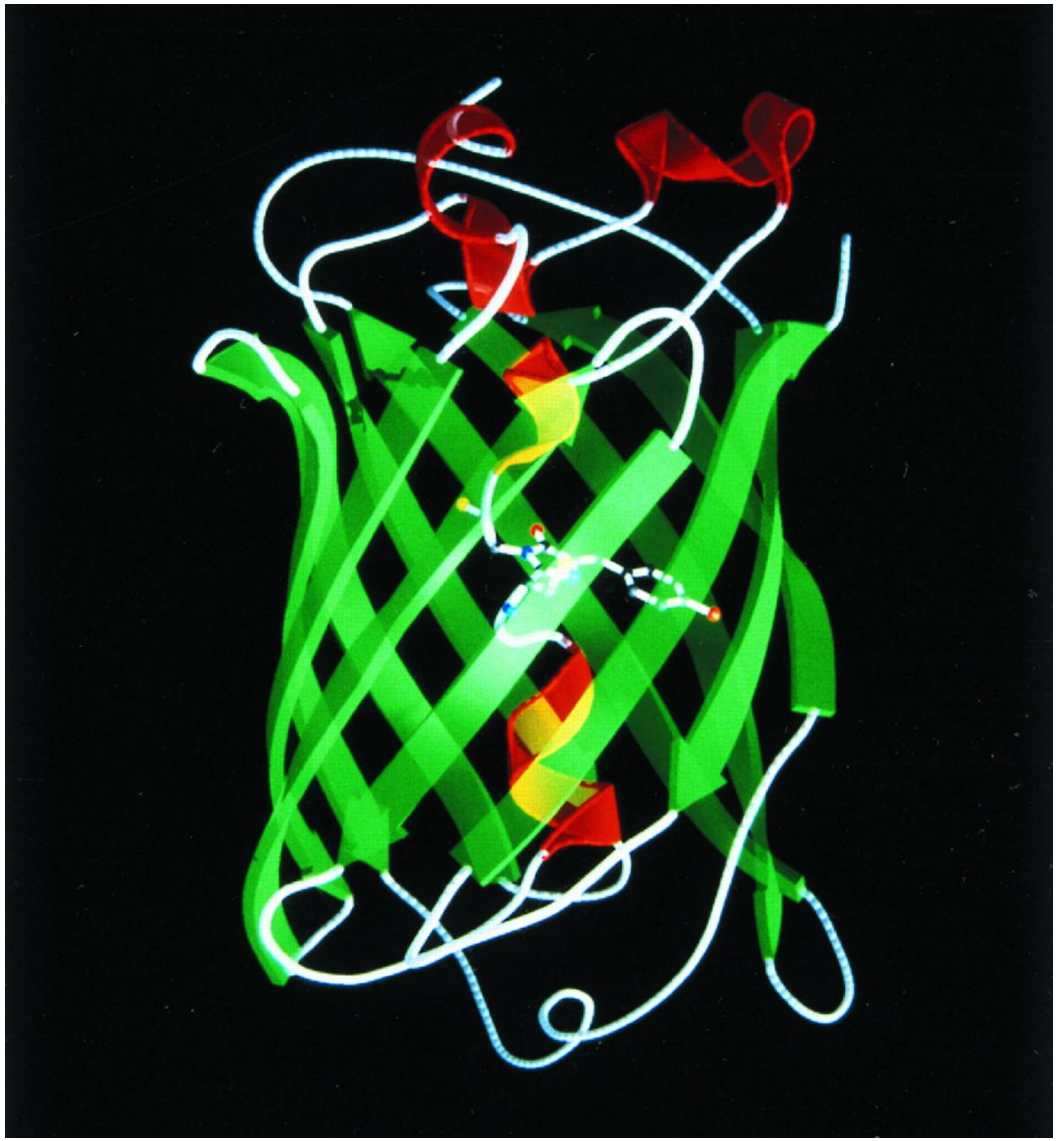
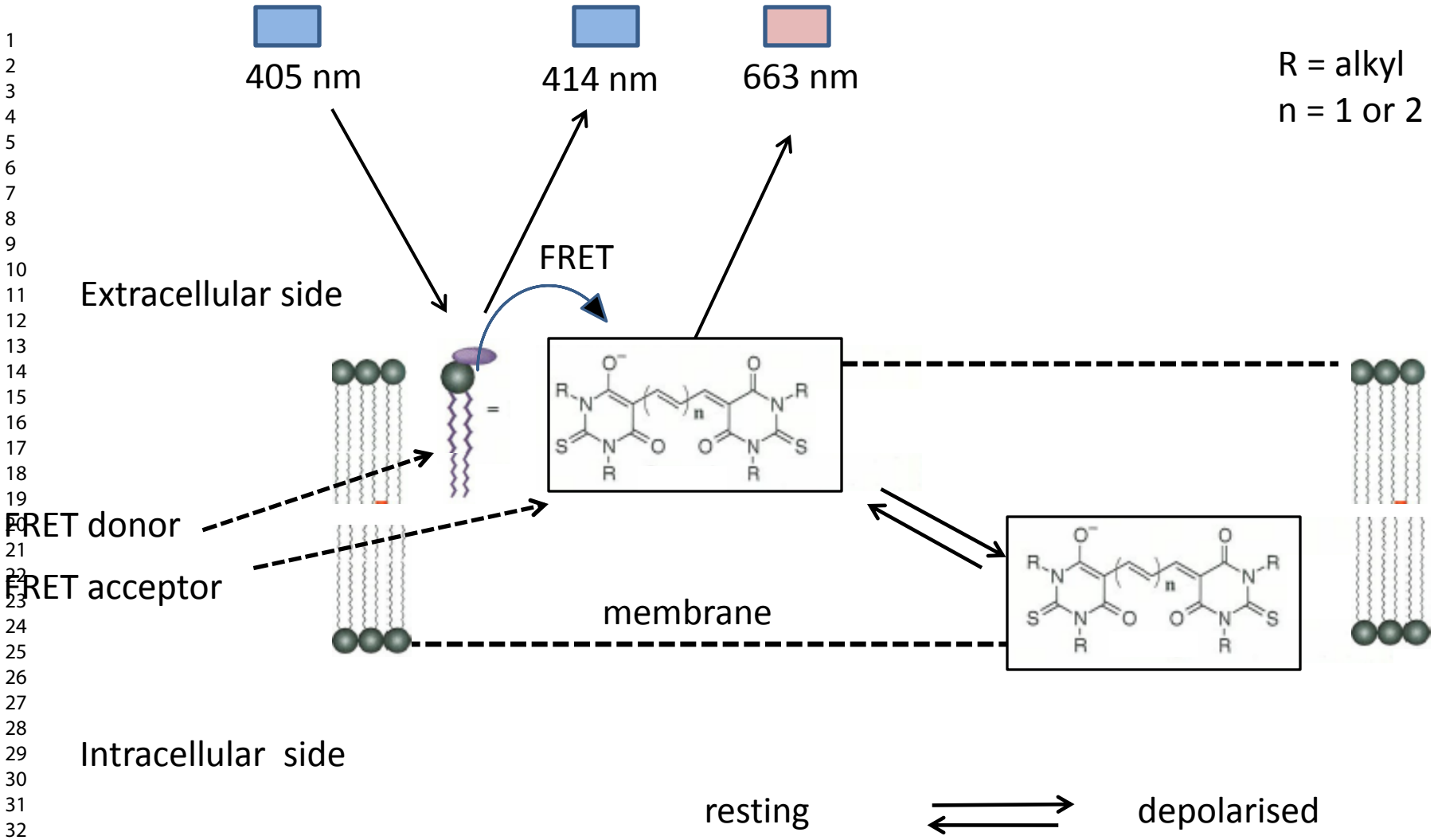


Figure 6

Figure 7




405 nm
414 nm
663 nm
R = alkyl
n = 1 or 2
FRET
Extracellular side
FRET donor
FRET acceptor
membrane
Intracellular side
resting
depolarised

Author photograph